THE CHANGE IN THE PHOSPHOPROTEINS OF THE YOLK DURING THE DEVELOPMENT OF THE CHICK EMBRYO

by

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INTRODUCTION

The yolk of the hen's egg is a rich source of phosphoproteins. Because the egg is readily available, it is a logical source of material for the study of these proteins. However, the yolk proteins now characterized have been shown to consist of several components and appear to be groups of similar proteins rather than single homogeneous compounds.

> No protein of egg yolk has been shown to have been isolated in pure form by the criteria currently applied. Knowledge of the composition of the lipoprotein, the phosphoprotein, and the livetin fractions of egg yolk is incomplete, and none of the individual yolk proteins is adequately characterized. (Fevold, 11).

In addition, there would seem to be a dearth of information on the change in the proteins during the development of the chick embryo in the egg. All research work has been concentrated on studies of the amino acid, lipid, and mineral contents of the yolk during embryo growth, and any conclusions concerning protein variations have been made from this information. There have been no studies on the changes in the proteins as entities.

A major reason for such a lack of information has been the complexity of available methods and the difficulties and inaccuracies involved. The recent development of electrophoretic analysis, however, provided a new method for protein investigations. Electrophoretic examination revealed the heterogeneity of the yolk proteins which were previously considered pure, and at the same time demonstrated the potential value of electrophoresis in yolk protein studies. Radioactive

isotopes were introduced as tracers in yolk phosphorus studies. Consequently, both of these techniques were combined and adapted for use in investigations on the phosphoproteins of egg yolk.

The development of this new method made possible the simultaneous observations of the yolk proteins and their relative phosphorus content. The yolk phosphoproteins of the fresh egg were studied in this way. The opportunity for further work on these phosphoproteins and their relation to embryonic growth became apparent.

The following investigations were undertaken to determine any significant variations in the phosphoproteins of the yolk of the embryo during incubation, to reveal any relationships between these embryo yolk proteins and the proteins of the fresh yolk, and to develop the techniques involved in such investigations.

REVIEW OF LITERATURE

Investigations of the proteins of the egg yolk were first reported over one hundred years ago. Work in this field continued through to the twentieth century.[1] In 1900 vitellin was isolated and analyzed by Osborne and Campbell (26) and its phosphorus content was noted. Subsequently other workers, including Calvery and White (4), refined the precipi-

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[1]Extensive bibliographies on this early history can be found in Fevold (11), Needham (23), and Romanoff and Romanoff (28).

tation methods by which vitellin was obtained and established it as the protein part of a lipoprotein.

Similarly, Plimmer (27) isolated livetin, and Kay and Marshall (17) in 1928 reported that this protein contained no phosphorus and no lipid. In addition, the latter workers admitted that although the greater portion behaved by criteria adopted up to that time for protein characterization as a single individual, they were not sure it was a single protein.

In a more recent work, Alderton and Fevold (2) in 1945 developed a different procedure for the isolation of lipovitellin. Their product contained, besides this protein, phosphorus-containing substances which could be removed by solution in 10 per cent sodium chloride and precipitated by dialysis. The following year Fevold and Lausten (12) reported the isolation of a new lipoprotein which they called lipovitellenin. Lipovitellenin had twice as much lipid as lipovitellin and composed 40 per cent of total lipoprotein in yolk, but contained less than 1/3 as much phosphorus as lipovitellin. In 1948 Mecham and Olcott (21) gave a preliminary report of a phosphoprotein, which they had isolated, that contained at least 40 per cent of the phosphorus in the yolk proteins. The next year they announced this protein had been isolated in yield sufficient to account for at least 60 per cent of the yolk protein phosphorus (22), and named it phosvitin. Electrophoretic analysis of this protein in sodium citrate and sodium acetate buffers at PH 4.6 produced two boundaries, with mobilities in the citrate buffer of -12×10^{-5}

units for the larger boundary and $-9x10^{-5}$ for the smaller.

Shepard and Hottle (30) in the same year reported electrophoretic analysis of a preparation of yolk proteins, intended for use as an antigen, which they considered to be livetin. This solution was prepared by several different methods, but in all cases more than one boundary appeared during electrophoresis. In one procedure, these workers dialyzed their yolk preparation against 0.85 per cent sodium chloride at a low temperature, diluted it and adjusted it to a pH of 6. A flocculent precipitate formed and separated as a top layer in a separatory funnel. The bottom clear solution was drawn off and gave four, rather slow boundaries during electrophoresis. If this clear solution was first extracted with ether, the resulting preparation had four electrophoretic boundaries with mobilities of -14.96, -7.39, -4.54, and -2.51 ($x\ 10^{-5}$). They used a phosphate-sodium chloride buffer at a pH of 7.95.

In 1951, Young and Phinney (34) published results of investigations on the yolk proteins of salmon and hen eggs. They experimented with various methods of fractionation to obtain proteins of electrophoretic homogeneity but had success only in the case of lipovitellin from the hen's yolk. This protein, prepared by dialysis precipitation, gave a single boundary. The stock solution was also analyzed and gave three boundaries, containing 4.6, 10.2, and 85.1 per cent of the total material, in order of decreasing mobility. A sodium chloride-barbiturate buffer at pH 8.5 was used. The boundaries all developed in six hours of electrophoresis time,

and no further separation took place in an electrophoresis period of fifteen hours. No mobility values were reported.

It should be noted that, with the exception of Young and Phinney, few of these workers considered temperature conditions of the work important, although protein denaturation, always a factor to be considered, increases with increasing temperature. Also, with the exception of the stock solution of Young and Phinney, all preparations analyzed were separated by chemical precipitation methods, with the accompanying possibilities of denaturation of the proteins.

Radioactive phosphorus had been used in egg yolk investigations as early as 1938, when Hevesy and Hahn (16) first studied phospholipid synthesis by means of radioactive P^{32}. That same year, Entenman et al (10) published experimental findings on the deposition of radioactive phospholipid in egg yolk after subcutaneous injections of P^{32} into laying hens. Radioactive phospholipid was found in all yolks from birds killed six and twelve hours after injections, illustrating the rapid incorporation of the phosphorus into the yolk compounds.

Hevesy (15) investigated the utilization of inorganic radioactive phosphorus in the yolk of the embryo, and from his results concluded all yolk phosphorus was converted into inorganic phosphorus before it was utilized by the embryo. Chargaff (6) reported studies on the relative rates of formation of egg yolk phosphorus compounds using the P^{32} as the tracer. He determined that in yolks from eggs laid during the first five days following injection of radioactive sodium

phosphate into laying hens, the vitellin exhibited a higher activity than the phosphatides. Lorenz, et al (19), in similar experiments, found that the white yolk takes several months to develop, but the deposition of the yellow yolk is complete in seven to ten days.

Driggers et al (9) hatched chicks from eggs laid by hens that had been fed both P^{32} and Ca^{45}. They found that the greatest concentration of radioactive phorphorus was found in the chicks from eggs laid six days after administration when only one feeding of the radioactive phosphorus had been given. All the results indicated that both isotopes were incorporated in the tissues in direct proportion to the total element assimilated, showing that there was no differential use of the isotopes. The report by Hevesy (15) fits in well with these results.

Electrophoretic analysis and radiotracer methods were combined by Hein and Clegg (14), who developed a modified electrophoresis cell that made it possible to measure the radioactivity associated with labeled phosphoproteins as the protein migrated past a point during an electrophoresis. Clegg and Hein (7) in this manner studied the phosphorus distribution in the serum proteins of the chicken.

Clegg et al (8) subsequently developed a method for preparing clear solutions of fresh egg yolk proteins, from which only ether extractable materials were removed. In this investigation all of the experimental work was carried out at low temperatures, 5°- 7°C., to keep protein denaturation to a minimum. The workers used this method to prepare solutions

of yolks from eggs laid by hens fed P^{32}, and analyzed these solutions employing the electrophoresis-tracer techniques. Two buffers, a sodium tetraborate and sodium citrate buffer of pH 7.5 and a glycine and disodium phosphate buffer of pH 9.4, were used. In both cases four major boundaries were observed, although the two electrophoretic patterns differed. In both buffers, a fast-moving component, containing about 39 per cent of the phosphorus of the preparation but only about 11 per cent of the total area, could be distinguished. These workers concluded, from a comparison of the data of the two buffer systems, that the differences between the patterns and activities of the three slower components might indicate a lack of homogeneity in some of these components.

There is no record of investigations on the yolk proteins of the developing chicken embryo that can be compared to the reported work on fresh yolk proteins. There are only a few scattered reports concerning any variations of definite proteins in the embryo yolk during incubation, although the change in the nitrogen, phosphorus, lipid, carbohydrate, and amino acids of the yolk during embryonic development has been studied (23, 24, 25, 28, 33). In an elaborate study, Schenck (29) isolated proteins he termed vitellin, albumin, and globulin from embryo yolks and determined their constituent amino acids. From his results he concluded that part of the original proteins were utilized as larger fragments, part as smaller building units in protein synthesis, and part disappeared completely and must have been used for energy. Schenck decided that the proteins

in the yolk change in composition during embryonic development. Needham (24), however, considered Schenck's work of little value, in that the differences in analytical results upon which Schenck's conclusions were based were within the allowed experimental error of his methods. Nevertheless, it is considered probable that the proteins of the yolk are broken down to some extent before the chick can use them and are not incorporated into the embryo as the original molecules (Needham, 25). Yolk protease activity has been noted which reached a maximum about the tenth day of development (Needham, 24).

MATERIALS AND METHODS

For a study of protein changes in the yolk of the chick during embryo development, certain stages in embryonic growth must be chosen, for continuous observation would be impractical. After a consideration of the literature, the stages of development at the end of seven, fourteen, and nineteen days of incubation of the eggs were picked, since previous investigators have found minima and maxima absorptions of nitrogen, amino acids, and lipids from the yolk at or about these times (24, 33, 23).

The liquid yolks taken from embryos at these times were submitted to the same preparatory treatment as the fresh yolks described by Clegg et al (8), with such modifications as required by slight differences in the properties of the embryo yolk material.

Production of Radioactive Eggs

The eggs used in the experiments were laid by two hens each fed 0.20 millicuries of P^{32} daily for twenty-two days. The P^{32} was obtained from Oak Ridge National Laboratories in the form of a phosphate salt in weak hydrochloric acid, and was put in oil-coated, gelatin capsules and force-fed to the hens.

The hens were kept in wire, laying cages in a hood in one of the radiological laboratories. The back and sides of the hood and the trays placed below the cages were lined with oilcloth. All droppings, spilled feed and water, and feathers fell on the trays, which were emptied frequently and relined with fresh oilcloth. The wastes and dirty oilcloth were placed in cardboard boxes and kept isolated at -17°C. until the radioactivity fell to prescribed levels, when it was disposed of by burial in ground used specifically for such purposes. All personnel having direct contact with the hens, and the persons concerned with the handling of the phosphorus solution wore rubber gloves and some protective body covering (laboratory coat or apron). A protective cover for the hands was always used when the eggs were handled. At the end of the twenty-two days the hens were killed and placed in cardboard boxes along with the wastes.

The eggs were fertilized by artificial insemination of the hens at three or four day intervals. The eggs laid during the first six days of P^{32} feeding were not used in the study, since

at least that period of time was required for the concentration of radioactive phosphorus in the yolk and its proteins to become appreciable (Lorenz, et al 19). The eggs were stored in cardboard cartons in the refrigerator, 7°- 10°C., until they were placed in the incubator. They were set in a Humidaire incubator, model 55, in lots of four or five at three to four day intervals, and incubated at a temperature of 99°- 100°F. with a relative humidity of 85 - 87 per cent. At the end of seven, fourteen, and nineteen day periods the eggs were candled to determine their fertility, and fertile eggs of the desired incubation time were removed from the incubator and placed in the refrigerator.

Extraction and Preparation of the Yolk Proteins

The cooled eggs were placed in iced, distilled water and the shell and chorionic membrane opened and removed from around the embryo. Various methods, depending upon the age of the embryo, were employed to separate the yolk from the yolk sac. The yolk material of the seven-day embryo was extracted from the sac with a hypodermic syringe while the embryo was under water. The fourteen-day embryo yolk material was obtained with the syringe after the amniotic membrane was cut away under water and the embryo with yolk sac intact was taken from the water and placed on a clean cloth. In the case of the nineteen-day embryo, the amniotic membrane was ruptured, the embryo was removed to a clean cloth, and then the embryo was held above a graduated cylinder and funnel while the yolk sac

was slit. The yolk material would drip through the funnel into the graduated cylinder. During this procedure the fine, solid, yellow material lining the yolk sac at this age was prevented, in so far as possible, from being carried along with the liquid yolk.

The contents of the yolks were collected in 100 millliliter glass-stoppered graduated cylinders and diluted 1:6 with a cold, 15 per cent glycine solution, pH 7.5. The yolk and glycine solution were carefully mixed until a homogeneous suspension was obtained. All subsequent procedures were carried out in a cold room, 5° - 10°C.

Twenty-five to 50 ml. portions of the yolk suspensions were transferred to Erlenmeyer flasks and two volumes of cold, reagent grade ether, saturated with water, were added. The mixtures were carefully swirled to insure mixing and allowed to stand for one to two hours. Most of the ether was poured off and the process repeated, six times in all. The final extraction was completed in a separatory funnel, and all of the solution except the top ether layer was drawn off and transferred to a dialysis tube, which was subsequently placed in a solution containing 15 per cent glycine and 10 per cent sodium chloride. Dialysis was allowed to proceed until an appreciable amount of ether had collected at the top of the solution. This ether was removed and the procedure repeated until no more ether separated from the protein solution; it was then dialyzed for twenty-four hours more, at which time a clear or opalescent solution was obtained.

The solutions were then dialyzed, with frequent changes, against the following buffers:

(1) Glycine-phosphate: 0.053 M. disodium phosphate and 0.05 M. glycine, adjusted to pH 9.4 with sodium hydroxide. This buffer was the one used in fifteen of the eighteen experiments carried out.

(2) Borate-citrate: 0.05 M. sodium tetraborate and 0.10 M. sodium citrate adjusted to pH 7.5 with citric acid. This was used for only three nineteen-day embryo yolk solutions.

The glycine-phosphate buffer was used in the majority of the experiments because it gave a better electrophoretic separation of the protein solution than the borate-citrate, and in a shorter length of time. However, with the nineteen-day embryo yolk solutions it was difficult to obtain solutions clear enough for electrophoretic analysis when the glycine-phosphate buffer was used in the dialysis; the borate-citrate gave clearer solutions and therefore was employed in the dialysis and electrophoresis of three of the nineteen-day yolks.

The dialysis was carried out until the solutions were clear or until it was apparent that there was no precipitate settling out of the solutions. The solutions were subsequently used for electrophoretic and radiotracer analyses.

Electrophoresis and Tracer Techniques

The electrophoretic experiments were carried out in a Tiselius apparatus as modified by Longsworth (18), made by the Klett Manufacturing Company of New York. The operation of this apparatus at Kansas State College has been described by McColloch (20), Stanley (31), and Brandt (3). Simultaneously with the electrophoresis experiments, observations of the radioactivity of the protein components in a boundary of the solution under analysis were made. The activity of the P^{32} incorporated in the phosphoproteins of the yolk was followed in the descending boundary, using a modification of one of the arms of the center cell of the electrophoresis apparatus, according to Hein and Clegg (14) and Clegg and Hein (7). A semicylinder was ground in one of the arms of the cell about 1/3 of the distance from one end. The minimum thickness of the glass at the resulting "window" was 0.003 - 0.004 cm. For the counting data, an Eck-Krebs type counter tube with a diameter slightly less than that of the semicylinder ground in the cell was used. It was held in the desired horizontal position close to the thin "window" by means of a Lucite clamp and rubber bands. The counter tube was connected to a Berkeley 100 scaler.

At the beginning of each experiment, the electrophoretic cells, rack, and assembly were placed in the cold room and allowed to remain until cool. The yolk protein solution was removed from the dialysis tube through a puncture made near the

bottom of the tube and allowed to run into a beaker. The protein material was filtered through Whatman # 50 filter paper to prevent any small particles of tissue or other foreign matter that might be present from being carried along with the solution. The cell was filled with the solution according to the standard procedure (3, 20, 31).

The complete assembly was taken to the electrophoresis apparatus and immersed in the constant temperature water bath. For some of the experiments the temperature of the bath was maintained at 0.5°C., but because of difficulties experienced during the summer weather with the operation of the control system at this temperature, many of the runs were done at a temperature of 4°C.

The counter tube was then placed in its position next to the cell, and the scaler was put into operation. The boundaries were moved into view and the current applied, according to the usual procedure followed in carrying out an electrophoresis. Radioactivity was observed on the scaler during the course of the run during five or ten minute periods, usually consecutive, and recorded on forms designed for that purpose. Notations of pertinent conditions, such as bath temperature, and sketches of the developing boundary pictures and corresponding points of significant radioactivity were also recorded on this form. Since the descending boundary movement was under observation, the radioactivity at the beginning of the electrophoresis was that of the entire solution and the maximum disintegration rate recorded. As the boundaries separated and

moved past the "window" the radioactivity decreased in proportion to the amount of P^{32} present in each boundary, and at the end of the experiment, when the buffer solution was at the window level, the background radioactivity was observed. In this way the average radioactivity in each boundary could be determined and the percentage of P^{32} in each component calculated.

Visual observation of the electrophoresis pattern was made using the cylindrical lens system, but all photographs were taken employing the schlieren lens system. Both the descending and ascending boundaries were photographed on Eastman-Kodak Metallographic plates, or, when the solution was too opaque to give completely distinct boundaries, on Kodak Spectroscopic plates.

After the boundary pattern exhibited a satisfactory separation of its components, and photographs had been taken, the boundary was moved manually past the "window". Motion was halted at points of component division and several activity readings recorded; the moving of the boundary was then resumed, and the procedure continued until the buffer solution was in front of the "window."

The mobilities were calculated by the standard method [Brandt (3)], using the basic equation $\mu = \frac{v\ q\ k}{i}$ where μ is the mobility in $cm.^2 sec.^{-1}\ volt^{-1}$, v is velocity of migration of the peak concerned in cm./sec., q is the cross-sectional area of cell in $cm.^2$, k is the specific conductance in mho/cm., and i is the current in amperes.

Analytical Procedures

The protein solution remaining after each electrophoresis in which the glycine-phosphate buffer was used was dialyzed against the borate-citrate buffer. This buffer was changed at intervals until presumably all of the glycine-phosphate buffer was removed. The protein solutions were stored in glass test tubes sealed with tinfoil-covered cork stoppers. Samples of the dialysis solution were stored with them to serve as blanks.

Nitrogen, phosphorus, and lipid analyses of the solutions were made. Nitrogen was determined using a micro-Kjeldahl method as described by Hawk et al (13), pp. 820-822. Copper sulfate was used as the catalyst in the digestion, and several drops of hydrogen peroxide were used to speed oxidation. For the distillation, five milliliters of a 4 per cent solution of boric acid was used in the receiving flask. A mixed indicator, made up of 0.03 per cent methylene blue and 0.1 per cent methyl red in a 20:3 ratio, was employed in the subsequent titration with 0.01 N. sulfuric acid.

The total phosphorus was determined colorimetrically by the method of Fiske and Subbarow as described by Hawk et al (13), p. 583.

The lipid analyses were carried out as follows: five milliliters of the protein solution were pipetted into a small beaker and about ten milliliters of concentrated nitric acid were added. This mixture was placed under a heat lamp in the hood and evaporated to dryness; another five milliliters of

the concentrated nitric acid were added and the solution evaporated once more. This process was continued until the solution was clear, and the solution was evaporated to dryness for the last time. When cool, the residue was carefully treated with distilled water and dissolved to a great extent. Purified hexane was added to dissolve the lipid material, the mixture was gently swirled, and all was transferred to a test tube. The beaker was rinsed several times with hexane and the rinsings were added to the test tube. The top layer of hexane separated and was removed with a small medicine dropper to a previously weighed dish. More hexane was added and this process was repeated. The hexane was allowed to evaporate, and the dish and lipid material dried briefly under the heat lamp. When cool, the dishes were weighed and the weight of the lipid calculated.

RESULTS AND DISCUSSION

A total of eighteen experiments were carried out on the embryo yolk proteins using the electrophoresis-tracer methods. Five of these experiments were concerned with yolks from embryos of seven days' incubation; three were successful. Six were carried out with yolks of embryos of fourteen days' incubation, and five of these proved successful. Seven with yolks of embryos of nineteen days' incubation were carried out. Four of these were with the glycine-phosphate buffer, and resulted in only one successful experiment. Three were with the borate-citrate buffer, and gave two successful sets of

data. The unsuccessful experiments were the results of: (1) protein solutions with such a high degree of opacity that no distinct pattern photographs could be obtained, and (2) operational failures in the counting equipment.

The per cent of area (component), as determined by the method of Tiselius and Kabat (32), and the per cent of phosphorus in each peak in the seven, the fourteen, and the nineteen day yolk electrophoretic patterns, are given in Tables 5, 6, and 7, respectively (Appendix). These results are graphically illustrated in Plates II, III, and IV.

In addition to the eighteen experiments mentioned above, mobility determinations were carried out with non-radioactive yolk proteins from embryos of the same ages. The resulting mobilities were correlated with those of the radio-chemical experiments and the two sets of values proved to be very close. These mobilities are recorded in Table 1.

Analyses of each of the eighteen solutions were made. The results of the phosphorus, nitrogen, and lipid determinations are given in Tables 2, 3, and 4. These values, as determined on the yolk proteins prepared for electrophoresis, are given in milligrams per milliliter of the solution. Since the values depend upon the dilution of the original yolk samples, they are significant only as a measure of the relative concentration of the different substances. The ratios between the values of the three respective substances for each sample were calculated to obtain significant data. These ratios may be used for direct comparison, since dilution is no longer a

Table 1. Mobilities of embryo yolk proteins in glycine-phosphate buffer, pH 9.4, ($-\mu \times 10^5$)

Seven Days' Incubation Fraction				Fourteen Days' Incubation Fraction			Nineteen Days' Incubation Fraction	
1	2	3	4	A	B	C	X	Y
14.7	9.0	5.8	4.0	13.7	7.0	4.1	6.3	3.4
14.7	9.0	5.9	3.7	13.6	7.9	4.5	6.7	3.5
14.7	9.3	6.6	4.6	13.7	7.6	5.1	6.5	4.1
				13.7	7.3	5.3		
				13.7	9.2	6.5		

Table 2. Analytical results of the seven-day embryo yolk preparations

Nitrogen mg./ml.	Phosphorus mg./ml.	Lipid mg./ml.	P/N	P/Lipid	Lipid/N
1.79	0.362	2.1	0.20	0.17	1.2
1.31	0.230	1.2	0.18	0.19	0.9
1.81	0.253	1.6	0.14	0.16	0.9
1.48	0.180	1.4	0.12	0.13	0.9
1.06	0.232	1.1	0.22	0.21	1.0
Averages 1.49	0.251	1.5	0.17±.03	0.17±.02	1.0±.1

Table 3. Analytical results of the fourteen-day embryo yolk preparations

Nitrogen mg./ml.	Phosphorus mg./ml.	Lipid mg./ml.	P/N	P/Lipid	Lipid/N
0.56	0.171	0.9	0.31	0.20	1.5
1.03	0.164	1.5	0.16	0.11	1.4
0.94	0.163	0.8	0.17	0.20	0.9
0.57	0.224	1.1	0.39	0.20	1.9
0.87	0.273	2.1	0.31	0.13	2.4
0.76	0.205	1.5	0.27	0.14	2.0
Averages 0.79	0.200	1.3	0.27±.07	0.16±.04	1.7±.4

Table 4. Analytical results of the nineteen-day embryo yolk preparations

Nitrogen mg./ml.	Phosphorus mg./ml.	Lipid mg./ml.	P/N	P/Lipid	Lipid/N
0.45	0.023	0.6	0.05	0.04	1.3
0.54	0.058	0.5	0.11	0.12	0.9
0.38	0.058	0.5	0.15	0.12	1.3
0.52	0.068	0.3	0.13	0.23	0.6
0.69	0.094	0.9	0.14	0.10	1.3
0.72	0.064	0.2	0.09	0.32	0.3
0.72	0.055	0.6	0.08	0.09	0.8
Averages 0.57	0.060	0.5	0.11±.03	0.15±.08	0.9±.3

factor. The lipid referred to here should be considered bound in some manner, as it was not ether extractable.

In the electrophoresis pattern of the seven-day embryo, four major peaks were evident (B in Plate I). The pattern resembled closely the pattern of the fresh egg yolk proteins (A in Plate I). The peak with the highest mobility had an average area of 17 per cent of the total and contained an average of 41 per cent of the total phosphorus of the material. It had a mobility of -14.7×10^{-5}. The second peak had 8 per cent of the area and 6 per cent of the phosphorus, with a mobility of -9.0×10^{-5}. In the third peak there was 14 per cent of the area and 9 per cent of the phosphorus, and this peak had a mobility of approximately -5.8×10^{-5}. The fourth and last peak contained 57 per cent of the total area, 42 per cent of the total phosphorus, and its mobility was about -4.0×10^{-5}. Three per cent of the area and 2 per cent of the phosphorus activity appeared in the salt boundary.

Consequently, the first, fast peak contained a comparatively large amount of phosphorus. Although the first and fourth peaks contained about the same percentage of total phosphorus, the fourth peak had three times as much material in it. This comparison is illustrated in Plate II. The first peak, therefore, had about three times the phosphorus concentration of the fourth peak. These two peaks appeared to be the major phosphorus-containing components.

The protein solution from the fourteen-day embryo yolks separated during electrophoresis into three boundaries, C in

Plate I. The fastest peak, containing an average of 9 per cent of the total area, had 28 per cent of the total phosphorus, and had a mobility of -13.7×10^{-5}. The second peak had 22 per cent of the area and 12 per cent of the phosphorus, with a mobility of -7.0 - -7.9×10^{-5}. The third peak has 67 per cent of the total area, 58 per cent of the total phosphorus, and a mobility of -4.1 - -4.5×10^{-5}. The salt boundary accounted for about 2 per cent of both the total area and total phosphorus activity.

The material in the fastest boundary contained the highest concentration of phosphorus, since it represented only 9 per cent of the proteins, but had almost 1/3 of the total phosphorus. The third boundary had seven times as much material in it as the first boundary, but had only about twice as much phosphorus. These relationships are shown in Plate III.

The nineteen-day embryo yolk protein solutions exhibited two electrophoretic boundaries in both of the buffers used. That in the glycine-phosphate buffer is D of Plate I. Only one solution in the glycine-phosphate buffer was clear enough to give a picture for area determinations. Two solutions in the borate-citrate buffer gave suitable photographs. In the glycine-phosphate buffer, the first boundary, with a mobility about -6.5×10^{-5}, had 65 per cent of the area and 29 per cent of the phosphorus. The second boundary had 35 per cent of the area and 71 per cent of the phosphorus, and its mobility was -3.4 - -3.5×10^{-5}. A close examination of this second boundary revealed an indication that it actually might consist of two peaks, but further resolution was never obtained, so it was considered as

EXPLANATION OF PLATE I

The electrophoretic patterns, descending boundaries, of yolk preparations from the fresh egg and from embryos. The glycine-phosphate buffer, pH 9.4, was employed.

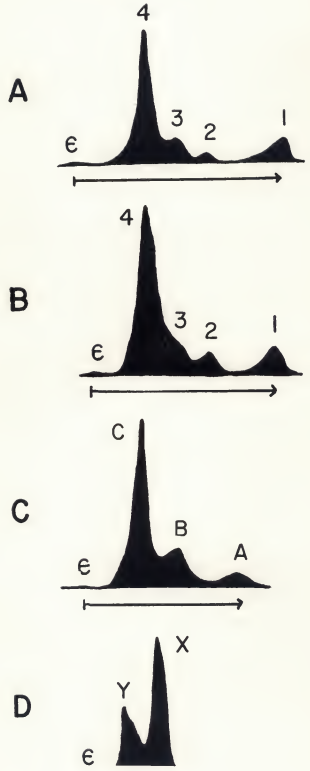
A. Fresh egg yolk, 150 minutes.

B. Yolk from embryo after seven days' incubation, 161 minutes.

C. Yolk from embryo after fourteen days' incubation, 116 minutes.

D. Yolk from embryo after nineteen days' incubation, 105 minutes.

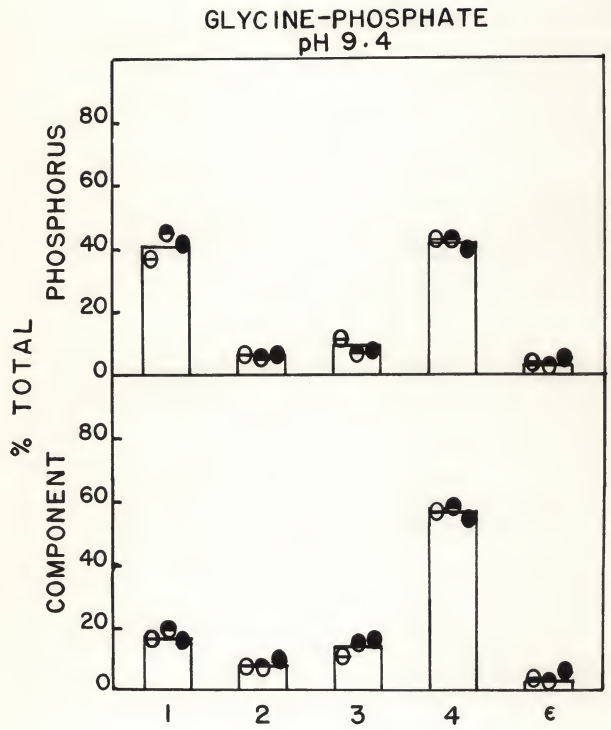
PLATE I



EXPLANATION OF PLATE II

Comparison of the percentage of the components present in the electrophoretic patterns of the seven-day embryo yolk preparations and the percentage of the phosphorus in the components.

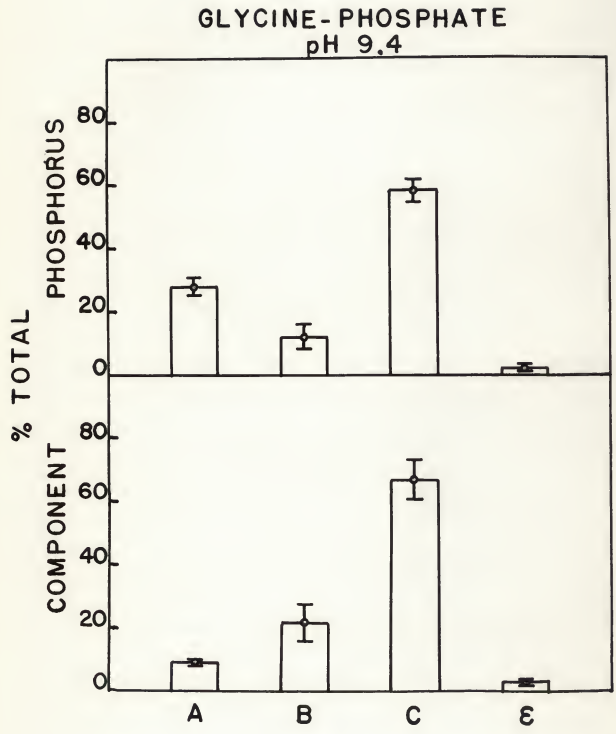
PLATE II


GLYCINE-PHOSPHATE
pH 9.4
% TOTAL PHOSPHORUS COMPONENT
80
60
40
20
0
80
60
40
20
0
1
2
3
4
ϵ

EXPLANATION OF PLATE III

Comparison of the percentage of the components present in the electrophoretic patterns of the fourteen-day embryo yolk preparations and the percentage of the phosphorus in the components. This is an average of five experiments.

PLATE III


GLYCINE-PHOSPHATE
pH 9.4
% TOTAL
PHOSPHORUS
COMPONENT
80
60
40
20
0
A
B
C
ε

one peak for the purpose of this discussion. The solutions in the citrate buffer gave boundaries of a similar pattern. Sixty per cent of the area and 32 per cent of the phosphorus were in the first boundary, and 40 per cent of the area and 68 per cent of the phosphorus were in the second boundary. The comparisons are shown in Plate IV.

Although the second boundary of the nineteen-day yolk solutions had twice as much phosphorus as the first and only 1/2 of the area of the first, this actually represented very little phosphorus. This is shown by the analytical results which will be discussed subsequently. It appears, therefore, that percentage comparisons at this age may not be significant.

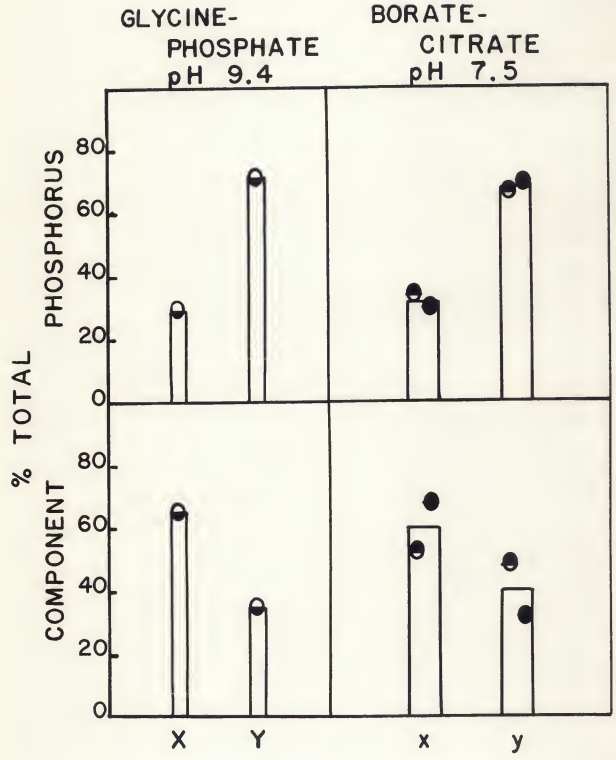
The average P/N ratio of the yolk preparations rose from 0.17 on the seventh day of incubation to 0.27 on the fourteenth day, indicating that the nitrogen in these preparations disappeared at a faster rate than the phosphorus during this period. From the fourteenth to the nineteenth day, however, this P/N ratio decreased to 0.11, suggesting a sharp decrease in the phosphorus relative to the nitrogen disappearance. From this information and from the low radioactivity associated with the nineteen-day yolk solutions, it might be concluded that much of the phosphorus has gone from the yolk proteins by the nineteenth day of incubation.

The phosphorus-to-lipid ratio, meanwhile, remained relatively constant from the seventh through the nineteenth days of incubation, with 0.17 at seven days, 0.16 at fourteen days, and 0.15 at nineteen days. It would appear that the

EXPLANATION OF PLATE IV

Comparison of the percentage of the components present in the electrophoretic patterns of the nineteen-day embryo yolk preparations and the percentage of the phosphorus in the components.

PLATE IV


GLYCINE-
PHOSPHATE
pH 9.4
BORATE-
CITRATE
pH 7.5
% TOTAL
PHOSPHORUS
COMPONENT
80
60
40
20
0
X
Y
x
y

same relative concentration is maintained throughout the incubation period by these substances. Another relationship, that between lipid and nitrogen, showed that the lipid behaved in the same way as the phosphorus towards the nitrogen.

It was observed that the yolk proteins of the embryo of seven days' incubation resembled those of the fresh yolk in electrophoretic pattern and relative amounts and phosphorus contents of the components, as reported by Clegg et al (8). In the seven-day embryo yolk proteins, two principal phosphoproteins that appear to be closely related, if not identical, to those in the fresh yolk were evident.

By the fourteenth day of development, only three electrophoretic boundaries appeared in the yolk proteins. The high value for the mobility and the high phosphorus content of the fastest-moving peak indicated some similarity between this component and the fastest phosphoprotein component of the seven-day yolk, but differences in the mobilities suggested they were not identical. There was only one middle peak in the fourteen-day embryo yolk electrophoretic pattern, in contrast to two middle peaks in the fresh and seven-day yolk protein patterns. Furthermore, this single peak had a much greater area than either of the two peaks of the earlier age, and a mobility ranging midway between the values of the two middle peaks of the seven-day embryo yolk proteins. This middle component contained a relatively small amount of phosphorus.

The last peak of the fourteen-day embryo yolk electrophoretic pattern had a mobility of -4.1 to -4.5 ($\times 10^{-5}$),

close to that of the last peak of the seven-day yolk proteins, with a mobility of about -4.0×10^{-5}, and also contained a large percentage of phosphorus.

On the nineteenth day of embryonal development, the electrophoretic pattern of the yolk proteins was greatly modified and had only two large peaks. These were rather slow-moving, with mobilities around -6.5 and -3.5 ($\times 10^{-5}$), respectively, with the latter containing the larger amount of phosphorus but the lesser area and indications that it might consist of more than one component. There was no evidence of the fast, high-phosphorus component present in all the other ages studied, and from the analytical data it was evident that the phosphorus content of the protein solutions dropped markedly between the fourteenth and nineteenth days of embryonal development.

Egg yolk proteins of high phosphorus content have been reported in the literature, (21, 22, 11, 23, 28). However, the methods and conditions of these previous investigations varied so widely that it is not possible to relate such proteins to those found in the embryo yolk in this work. Therefore, at this time, no attempt is made to relate phosphoproteins observed in these experiments with those recorded in the literature. It remains for further investigators to establish the relationships which might exist between the proteins lipovitellin, phosvitin, lipovitellenin, etc., and those shown present in the fresh yolk (8) and the embryonic yolk investigated in this study.

Also, the exact times of change in the yolk phosphoproteins during embryonic development should be determined. Of especial importance, the daily composition of the yolk between the fourteenth and nineteenth days of incubation should be studied. Whether the changes in the proteins are gradual or abrupt might be revealed by such investigations.

Another question to be answered by further experimentation is whether the changes are caused by enzymes present in the original yolk or enzymes from the embryo. Infertile eggs kept under the same conditions of incubation should provide a source of information in this respect and, in addition, the results might aid research on the nature of deterioration of stored eggs and egg products.

SUMMARY

The changes in the embryo yolk phosphoproteins were investigated using a combination electrophoresis-radiotracer method. Fertile eggs from hens fed radioactive phosphorus were incubated for seven, fourteen, and nineteen day periods. The liquid yolks from the embryos were suspended in a glycine solution, extracted with ether, and finally dialyzed against a glycine-phosphate or a borate-citrate buffer. These dialyzed solutions were analyzed by a combined electrophoresis-radiotracer technique. The nitrogen, phosphorus, and lipid (bound) content of these preparations was also determined, after they had all been dialyzed against the borate-citrate buffer.

Electrophoretic patterns of the seven-day embryo yolk

proteins closely resembled the patterns of those of the fresh yolk which had been obtained by other workers; four peaks were present. The component and phosphorus distribution in the boundaries were also similar. Two major phosphorus-containing proteins were apparent. The first had a relatively large amount of phosphorus and a high value for the mobility, -14.7×10^{-5}; the other had less phosphorus but composed about half of the total components present, and had a mobility of about -4.0×10^{-5}.

The fourteen-day embryo yolk preparations differed in electrophoretic pattern from the seven-day yolk proteins. Only three components were present, two of which contained appreciable amounts of phosphorus and appeared to be related to the phosphoproteins of the seven-day yolk. The peak containing a relatively large amount of phosphorus, had a mobility of -13.7×10^{-5}, and the phosphoprotein of greater area and less phosphorus content had a mobility of about -4.3×10^{-5}.

In the nineteen-day embryo yolk protein pattern, only two peaks were visible, although the slower of the two appeared to consist of two components which could not be resolved. No major phosphorus-containing protein similar to the fast-moving component of the earlier age embryo yolks was evident. The slower component of the nineteen-day yolk solutions had a mobility of -3.5×10^{-5} and contained the greater amount of phosphorus in the solution, suggesting some relationship with the slower phosphoprotein of the seven- and fourteen-day preparations.

Analytical results revealed that the nitrogen disappeared from the yolk proteins at a greater rate than the phosphorus or bound lipid during the first part of incubation, but this situation was reversed during the last days of embryonic development. Little of the original phosphorus was left in the yolk at the end of nineteen days of incubation.

No attempt was made to identify the phosphoproteins studied in this investigation with those characterized in the older literature. This task, along with greater and more detailed expansion of the studies on the embryonic yolk, will remain for further investigators.

ACKNOWLEDGMENT

The author wishes to express her appreciation to Dr. R. E. Clegg, her major professor, for his suggestion of this problem and his technical advice and assistance.

Thanks are also given to Dr. Ralph E. Silker, Head of the Department of Chemistry, who made it possible to obtain the necessary equipment for this investigation.

BIBLIOGRAPHY

(1) Alberty, Robert A.
Electrochemical properties of the proteins and amino acids. The proteins. Vol. 1, Part A. New York: Academic Press, 1953.

(2) Alderton, G. and H. L. Fevold.
Preparation of the egg yolk lipoprotein, lipovitellin. Arch. Biochem. 8:415-419. 1945.

(3) Brandt, Luther.
An electrophoretic investigation of the blood serum proteins of the common fowl and of certain gallinaceous hybrids. Ph.D. Thesis. Kansas State College. 1950.

(4) Calvery, H. D. and Abraham White.
Vitellin of hen's egg. J. Biol. Chem. 94:635-639. 1932.

(5) Chargaff, Erwin.
A study of lipoproteins. J. Biol. Chem. 142:491-504. 1942.

(6) _____
The formation of the phosphorus compounds in egg yolk. J. Biol. Chem. 142: 505-512. 1942.

(7) Clegg, R. E. and R. E. Hein.
P^{32} distribution in the serum proteins of the chicken. Science. 117:714-715. 1953.

(8) Clegg, R. E., R. E. Hein, C. H. Suelter, and R. H. McFarland.
The distribution of radioactive phosphorus in the electrophoretic components of egg yolk proteins. Poultry Science. 34: 210-214. 1955.

(9) Driggers, J. Clyde, Ray L. Shirley, George K. Davis, and N. R. Mehrhof.
The transference of radioactive Ca and P from hen to chick. Poultry Science. 30:199-204. 1951.

(10) Entenman, C., S. Ruben, I. Perlman, F. W. Lorenz, and I. L. Chaikoff.
Radioactive phosphorus as an indicator of phospholipid metabolism. III. The conversion of phosphate to lipoid phosphorus by the tissues of the laying and non-laying bird. J. Biol. Chem. 124:795-802. 1938.

(11) Fevold, Harry L.
Egg proteins. Adv. Prot. Chem. 6:188-246. 1951.

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(12) Fevold, H. L. and Adele Lausten.
Isolation of a new lipoprotein, lipovitellenin, from egg yolk. Arch. Biochem. 11:1-7. 1946.

(13) Hawk, P. B., B. L. Oser, and W. H. Summerson.
Practical physiological chemistry. 12th ed.
Philadelphia: Blakiston, 1947.

(14) Hein, R. E. and R. E. Clegg.
Modified electrophoresis cell for determination of labeled phosphoproteins. Nucleonics. 10:70-71. 1952.

(15) Hevesy, G.
Application of radioactive indicators in biology.
Ann. Rev. Biochem. 9:641-662. 1940.

(16) Hevesy, G. and L. Hahn.
Kgl. Danske Videnskab. Selskab., Biol. Medd. 14:1-39.
1938. Original not seen. In Romanoff and Romanoff.

(17) Kay, Herbert Davenport and Phillip Guy Marshall.
The second protein (livetin) of egg-yolk. Biochem. J.
22:1264-1269. 1928.

(18) Longsworth, L. G.
A modification of the schlieren method for use in electrophoretic analysis. J. Am. Chem. Soc. 61:529-530. 1939.

(19) Lorenz, F. W., I. Perlman, and I. L. Chaikoff.
Phosphorus deposition in the egg as measured with radioactive phosphorus. Am. J. Physiol. 138:318-327. 1943.

(20) McColloch, R. J.
An electrophoretic investigation of some pectic enzymes. Ph.D. Thesis. Kansas State College. 1948.

(21) Mecham, Dale K. and Harold S. Olcott.
An egg yolk protein containing 10% phosphorus.
Fed. Proc. 7:173. 1948.

(22) _____
Phosvitin, the principal phosphoprotein of egg yolk.
J. Am. Chem. Soc. 71:3670-3679. 1949.

(23) Needham, Joseph.
Chemical embryology. Three volumes. New York:
Macmillan, 1931.

(24) Needham, Joseph.
Biochemistry and morphogenesis. Cambridge: University Press, 1942.

(25) _____
Thoughts on the biochemistry of the developing hen's egg. Ann. Nutr. Al. 8:195-205. 1954.

(26) Osborne, Thomas B. and George F. Campbell.
The proteids of the egg yolk. J. Am. Chem. Soc. 22:413-422. 1900.

(27) Plimmer, R. H. Aders.
The proteins of egg-yolk. J. Chem. Soc. 93:1500-1506. 1908.

(28) Romanoff, Alexis L. and Anastasia Romanoff.
The avian egg. New York: John Wiley & Sons, 1949.

(29) Schenck, E. G.
Untersuchungen uber das Verhalten der Eiweisstoffe usw. Z. Physiol. Chem. 211:111-153. 1932.

(30) Shepard, C. C. and G. A. Hottle.
Studies of the composition of the livetin fraction of the yolk of the hen's egg with the use of electrophoretic analysis. J. Biol. Chem. 179:349-357. 1949.

(31) Stanley, William Gordon.
Electrophoretic studies of milk whey proteins. An investigation of the effect of the method of whey protein preparation and buffer solvent used on the electrophoretic pattern. Ph.D. Thesis. Kansas State College. 1952.

(32) Tiselius, A. and E. A. Kabat.
An electrophoretic study of immune sera and purified preparations. J. Exptl. Med. 69:119-121. 1939.

(33) Williams, Matthew A., William A. Da Costa, Lloyd H. Newman, a and Lawrence M. Marshall.
Free amino acids in the yolk during the development of the chick. Nature. 173: 490. 1954.

(34) Young, E. Gordon, and J. I. Phinney.
On the fractionation of the proteins of egg yolk. J. Biol. Chem. 193:73-80. 1951.

APPENDIX

Table 5. Results of electrophoretic and tracer data on seven-day embryo yolk protein solutions

(1) Component, Per Cent of Total Fraction				
1	2	3	4	ϵ
17	8	11	60	4
19	8	15	58	0
15	9	16	54	6
Averages				
17±1	8	14±2	57±2	3±2
(2) P^{32}, Per Cent of Total Fraction				
37	6	11	43	4
45	5	7	43	0
42	6	8	39	5
Averages				
41±3	6	9±2	42±2	3±2

Table 6. Results of electrophoretic and tracer data on fourteen-day embryo yolk protein solutions.

(1) Component, Per Cent of Total Fraction			
A	B	C	ε
11	24	66	0
9	31	57	3
8	26	63	3
10	14	73	3
9	13	74	4
Averages 9 ± 1	22 ± 6	67 ± 6	3 ± 1
(2) P^{32}, Per Cent of Total Fraction			
29	11	60	0
33	11	53	3
30	14	53	3
27	4	67	2
22	18	59	1
Averages 28 ± 3	12 ± 4	58 ± 4	2 ± 1

Table 7. Results of electrophoretic and tracer data on nineteen-day embryo yolk protein solutions.

	(1) Component, Per Cent of Total Fraction	
	X	Y
In glycine-phosphate	65	35
In borate-citrate	52	48
	68	32
Averages	60 ± 8	40 ± 8
	(2) P^{32}, Per Cent of Total Fraction	
In glycine-phosphate	29	71
In borate-citrate	34	66
	30	70
Averages	32 ± 2	68 ± 2

THE CHANGE IN THE PHOSPHOPROTEINS OF THE YOLK DURING THE DEVELOPMENT OF THE CHICK EMBRYO

by

JOAN JOHNSTON MULKERN

B. A., University of Kansas City, 1953

AN ABSTRACT

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1955

The object of this investigation was to adapt and extend an electrophoretic-radiotracer technique to the study of the phosphoproteins of the embryo yolk, to determine any significant variations in these phosphoproteins during incubation, and to reveal any relationship between these embryo yolk proteins and the proteins of the fresh yolk.

Fertile eggs laid by hens that were fed radioactive phosphorus were incubated for seven, fourteen, and nineteen day periods. The liquid yolks from the embryos were suspended in a glycine solution, extracted with ether, and finally dialyzed against a glycine-sodium phosphate or a sodium borate-sodium citrate buffer. These dialyzed solutions were analyzed by a combined electrophoresis-radiotracer technique. From this analysis, data were obtained on the percentage of total phosphorus and total component in each peak of the electrophoretic boundary of a solution, as well as information for mobility calculations. The nitrogen, phosphorus, and lipid (bound) contents of these preparations were also determined after the solutions had all been dialyzed against the borate-citrate buffer.

Electrophoretic patterns of the seven-day embryo yolk proteins closely resembled the patterns of fresh yolks which had been obtained by Clegg et al. Four peaks were present. The component and phosphorus distributions in the protein boundaries were also similar. Two main phosphorus-containing components were present; one had a mobility of -14.7×10^{-5} and a relatively large content of phosphorus, while the other

composed almost one half of the total material and had a lower relative phosphorus content and a mobility of about -4.0×10^{-5}.

The fourteen-day embryo yolk preparations differed in electrophoretic pattern from the seven-day solutions. Three components were present, two of which contained appreciable amounts of phosphorus and appeared related to the two phosphoproteins of the seven-day embryo yolk. The mobility of the fast component was -13.7×10^{-5}. The other had a mobility of about -4.5×10^{-5}.

In the nineteen-day embryo yolk solution pattern, only two peaks were visible, although the slower of the two appeared to consist of two components which could not be resolved. No major phosphorus-containing component similar to the fast-moving peak of the earlier age embryo yolks was evident. The peak containing the more phosphorus had the lesser area and a mobility around -3.5×10^{-5}. The other peak had a mobility of about -6.5×10^{-5}.

Analytical results suggested that the nitrogen disappeared from these yolk materials at a faster rate than the phosphorus between the seventh and fourteenth days of incubation. This was shown by a rise in the average P/N ratio from 0.17 to 0.27. This P/N ratio then dropped to 0.11 by the nineteenth day, indicating a relatively greater disappearance of the phosphorus during this period. The change in lipid values followed those of the phosphorus.

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